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Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source

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Abstract

Five obligate anaerobes that were most closely related to *Clostridium bifermentans*, *Clostridium celerecrescens*, *Clostridium saccharolyticum*, *Clostridium butyricum* and *Desulfovibrio desulfuricans* by their 16S rRNA genes sequences were isolated from enrichment cultures using hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source. The above isolates transformed RDX at rates of 24.0, 5.4, 6.2, 2.5, 5.5 μmol h^{-1} g (dry weight) of $cells^{-1}$, respectively, to nitrite, formaldehyde, methanol, and nitrous oxide. The present results indicate that clostridia are major strains responsible for RDX removal, and all isolates seemed to mainly transform RDX via its initial reduction to MNX and subsequent denitration. Since clostridia are commonly present in soil, we suggest that they may contribute to the removal of RDX in the subsurface (anoxic) soil.
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1. Introduction

Wide military and civilian application of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a highly explosive compound, has resulted in severe soil and groundwater contamination [1,2]. RDX is toxic to various terrestrial and aquatic species [3,4]. The energetic chemical is a highly oxidized molecule that tends to be reduced under anaerobic conditions [5–22,27–29,31,33]. Most reported RDX-degrading anaerobic bacteria are facultative including members of the *Enterobacteriaceae* family (*Klebsiella pneumoniae*, *Serratia marcescens*, *Morganella morganii*, *Citrobacter freundii*, and *Escherichia coli*) [10,11]. None of above bacteria was reported to grow on RDX as a nitrogen source.

Although obligate anaerobes always exist in RDX-degrading anaerobic consortia, little is known about the role of obligate anaerobes in degradation of the energetic chemical. Adrian et al. [6–8] and Beller [12] proposed that acetogens are responsible for RDX removal in anaerobic consortia, but no acetogenic species were isolated. Boopathy et al. [13] described degradation of RDX by a sulfate-reducing consortium from creek sediment, but degradation of RDX by an individual sulfate-reducing bacterium was not reported. Thus far only one obligate anaerobe, *Clostridium bifermentans*, isolated from a contaminated soil, was reported to remove RDX in a complex brain-heart infusion medium [19].

One major concern regarding anaerobic degradation of RDX is the potential accumulation of toxic nitroso derivatives [5–11]. We previously found that an anaerobic sludge [16–17] and a facultative anaerobic isolate, *K. pneumoniae* strain SCZ-1 [18], were able to cleave the RDX ring to ultimately give nitrous oxide (N_2O), formaldehyde (HCHO) and methanol (CH_3OH). In the present study, our goal was to investigate the phylogenetic and metabolic diversity of RDX-transforming bacterial isolates in the strictly anaerobic mixed cultures enriched on RDX as a nitrogen source.

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2. Materials and methods

2.1. Chemicals and media

RDX (99% pure) was provided by Defense Research and Development Canada, Valcartier, Canada [23]. Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX, 99% pure) was synthesized according to the method described by Brockman et al. [25]. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, 98% pure) was provided by R.J. Spanggord from SRI International (Menlo Park, CA, USA). All other chemicals used were of reagent grade.

The basic salts and vitamins medium were prepared as described previously using a Wolin vitamin solution (5 ml in 1 l) and a trace metal solution [18,32]. The nutrient broth was composed of 3 g l^{-1} of beef extract and 5 g l^{-1} of peptone. Yeast extract (1 g l^{-1}), or bacto peptone (1 g l^{-1}) or glucose (1 g l^{-1}) was added to improve growth of bacterial isolates when necessary. The medium used for the isolation and maintenance of anaerobic bacteria was Bacto Brewer Anaerobic agar (58 g l^{-1}) (Becton Dickinson, Sparks, MD, USA).

2.2. Enrichment of anaerobic bacteria on RDX

The original anaerobic sludge was from a continuous upflow anaerobic sludge blanket digester (Biothane) (pH 6.5–7.5, 36–38°C), located in Sensient Flavor Canada (Cornwall, Ontario). It was used to convert nutrients in the food-processing wastewater to methane (70% of the total gas released). The following compounds were added to the basic salts and vitamins medium to enrich bacteria using RDX (0.1 mM) as a nitrogen source: hydrogen, formate and carbonate, ethanol, glucose and lactate (Table 1). Where applicable, sulfate (3.5 mM) was added to enrich sulfate-reducing bacteria. Prior to inoculation, the liquid medium in sealed serum bottles was degassed and charged with oxygen-free argon, followed by addition of sodium sulfide (0.025%) and L-cysteine HCl (0.025%). When RDX was used as the sole carbon and nitrogen source, the headspace was charged with hydrogen gas (1 atm). When formate and carbonate was used, the headspace gas was a mixture of carbon dioxide (20%) and hydrogen gas (80%). Nutrient broth was used as a rich medium to preserve all RDX-degrading bacteria in the anaerobic sludge.

One ml of original anaerobic sludge was added to 19 ml of anaerobic liquid medium containing RDX (0.1 mM) and incubated statically at 37°C. After RDX disappearance, a fresh amount of RDX (0.1 mM) was added and the microcosm was incubated for 2 weeks. The cultures were subcultured (5% transfer) consecutively seven times over a period of 6 months. The final enrichment cultures did not show any growth in the basic salts media in the absence of RDX (Table 1). The enrichment cultures were plated on Bacto Brewer Anaerobic agar prepared inside sealed serum bottles. Following incubation at 37°C (3–7 days), colonies with different morphologies were picked and were re-plated on the same agar media. The latter process was repeated three times for purification of isolates. Growth of the bacterial isolates on RDX, sodium nitrite ($NaNO_2$, 0.5 mM) and ammonium chloride (NH_4Cl, 0.5 mM) as nitrogen source was evaluated by measuring increase in OD_{600nm} and by microscopic examination.

2.3. Phylogenetic analysis of 16S rRNA gene sequences of bacterial isolates

Colonies grown on pre-reduced PY agar [30] were picked for extraction of total DNA and PCR amplification of 16S rRNA genes according to standard molecular biology methods [24,36]. Sequences, with a length ranging from 1236 to 1314 bases, of 16S rRNA were compared

Table 1
Growth of the anaerobic sludge enrichment cultures and biotransformation of RDX (0.2 mM) as a nitrogen source in the presence of different carbon and energy sources after 8 days of incubation under argon (measurements were done in triplicates with standard deviations in parentheses)

Carbon or energy source	Biomass increase (OD_{600nm})		RDX removal (%)	RDX products (% of total C or N)	
	Without RDX	With RDX		TNX	N_2O
H_2 (1 atm) (RDX as C source)[a]	0.01 (0.0)	0.07 (0.00)	94 (57)	0.1 (0.02)	31 (3)
Glucose (3.2 mM)[a]	0.05 (0.04)	0.55 (0.1)	98 (9)	0.4 (0.1)	0
Ethanol (11 mM)[a,c]	0.02 (0.01)	0.2 (0.1)	91 (8)	0.2 (0.05)	0.7 (0.8)
Ethanol (11 mM) and sodium sulfate (3.5 mM)[b,c]	0.03 (0.02)	1.0 (0.1)	78 (10)	0	21 (9)
Sodium formate (7.3 mM) and sodium carbonate (4.7 mM), H_2 (0.8 atm) and CO_2 (0.2 atm)[a]	0.01 (0.01)	0.06 (0.02)	59 (41)	0	28 (3)
Sodium lactate (13.5 mM)[b,c]	0.2 (0.1)	1.1 (0.1)	83 (0.6)	0	4.2 (5.8)
Sodium lactate (13.5 mM) and sodium sulfate (3.5 mM)[b,c]	0.3 (0.1)	1.3 (0.0)	93 (24)	0	0.2 (0.2)
Beef extract (3 g l^{-1}) and peptone (5 g l^{-1})[a,c,d]	0.6 (0.1)	0.6 (0.1)	98 (32)	0.9 (0.3)	0.08 (0.08)

[a]Initial biomass (OD_{600nm}) ranged from 0.02 to 0.06.
[b]Initial biomass (OD_{600nm}) ranged from 0.1 to 0.15.
[c]These enrichment cultures produced methane.
[d]The low biomass was probably due to conversion of carbon to methane.

to published sequences by BLAST. The 16S rRNA gene sequences of the isolates and those of closely related standard strains were aligned by ClustalX(1.81). The neighbor-joining method (in the MEGA2 package [26]) based on the pairwise nucleotide distance of Kimura 2-parameter was used to build the phylogenetic tree. The number of bootstrap repetitions was 1000.

2.4. Anaerobic transformation of RDX

General RDX biotransformation conditions are described in the above enrichment tests. The carbon and energy sources used for RDX transformation by enrichment cultures and their isolates are described in Table 1. For selected isolates, the headspace of serum bottles used for growth was charged with argon unless otherwise noted. Yeast extract (1 g l^{-1}), bacto peptone (1 g l^{-1}) or glucose (1 g l^{-1}) was added to improve growth of bacterial isolates when indicated. One ml of liquid culture was inoculated to 19 ml of liquid biotransformation media (initial OD_{600nm} of the 20 ml media after inoculation, 0.05–0.15). Microcosms were sampled under strictly anaerobic conditions for subsequent analyses as described below.

2.5. Analyses of RDX and its products

The concentration of RDX and its nitroso products MNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and TNX in the supernatants of the liquid samples were analyzed at 230 nm by a HPLC/UV method as described previously [16–18]. The methods for analyses of NO_2^-, CH_4, N_2O, HCHO and CH_3OH were described in earlier reports [16,18]. All tests were performed in triplicates.

3. Results and discussion

3.1. Enrichment of RDX-degrading bacteria from anaerobic sludge

In the enrichment cultures, optimal growth and RDX transformation was observed when glucose, ethanol, or lactate was added as a carbon source (Table 1). Addition of sulfate further improved growth on ethanol and lactate. The enriched mixed cultures removed 60–99% of 0.2 mM of RDX within 8 days, whereas in the abiotic controls with nitrogen in the headspace, RDX removal was less than 10%. Growth of bacteria on RDX in the presence of hydrogen gas or formate was poor. The entire final enriched mixed cultures showed little growth in the basic salts media in the absence of RDX (Table 1), indicating that the energetic chemical acted as a nitrogen source for growth.

All enriched mixed cultures transformed RDX with production of the nitroso derivatives MNX, DNX and TNX that did not persist (Fig. 1). Their disappearance was accompanied by the formation of HCHO, CH_3OH (data not shown) and N_2O (Table 1). The final yields of N_2O from RDX by five of the eight enrichment mixed cultures were very low (Table 1). Fig. 1 shows that N_2O was only formed as a transient product.

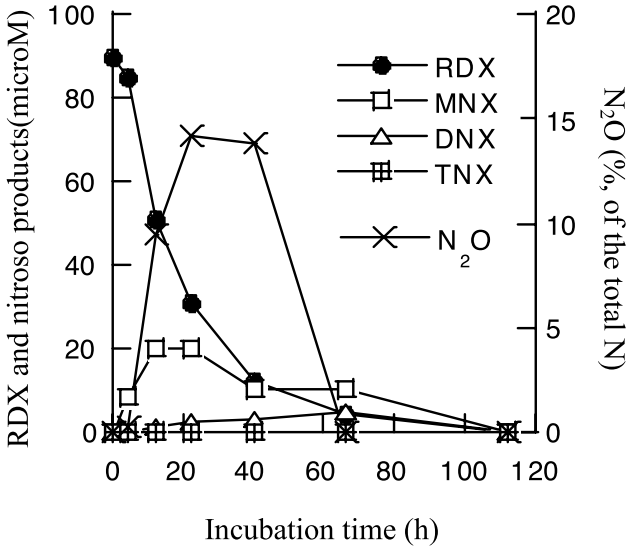

Fig. 1. N_2O production and removal during RDX biotransformation by the mixed cultures enriched on RDX and nutrient broth.

During RDX transformation with ethanol- or lactate-enriched mixed cultures, CH_4 was produced, but not in RDX transformation with glucose-enriched culture.

3.2. Phylogenetic diversity of RDX-transforming isolates in the enrichment cultures

Pure cultures were isolated and characterized from five of the above enrichment cultures using hydrogen, formate, glucose, ethanol or ethanol plus sulfate as co-substrate(s). Out of the 16 morphologically different colonies chosen, 15 exhibited RDX-removal activity. By comparing the partial 16S rRNA gene sequences of the isolates to gene in the GenBank, we found that the 15 colonies belonged to six genetically distinguishable bacterial species: HAW-1, HAW-G3, HAW-G4, HAW-E3, HAW-HC1, and HAW-ES2, isolated from enrichment culture fed with hydrogen, glucose, ethanol, formate, and ethanol plus sulfate, respectively.

All the isolates were catalase and oxidase negative, obligate anaerobes, five of which (HAW-1, HAW-G3, HAW-G4, HAW-E3, HAW-HC1) were Gram-positive, spore-forming, straight rods. The phylogenetic tree of 16S rRNA genes of the six isolates and those closely related standard bacterial strains is shown in Fig. 2. The sequences of 16S rRNA genes of the five Gram-positive isolate (HAW-1, HAW-G3, HAW-G4, HAW-E3, HAW-HC1) fell within the clusters of *Clostridium* genus. Collins et al. found that all the known species of *Clostridium* genus

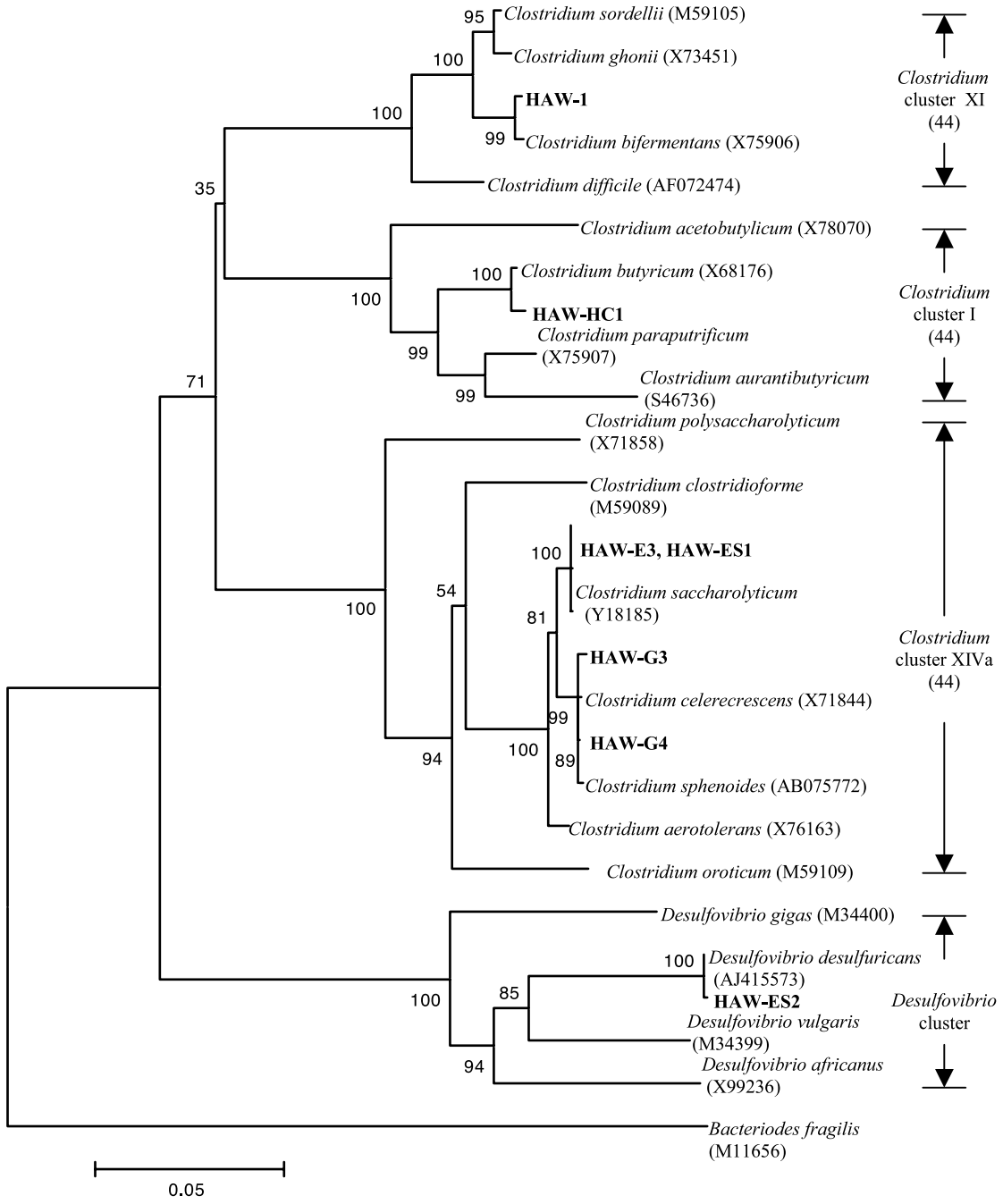

Fig. 2. Phylogeny of anaerobic bacterial isolates. The phylogenetic tree was generated based on the pairwise nucleotide distance of Kimura 2-parameter using the neighbor-joining method included in MEGA2 software package. The bar indicates the difference of two nucleotides per 100. The number beside the node is the statistical bootstrap value.

were very heterogeneous by their 16S rRNA gene sequences, and could be separated to 19 clusters [44]. HAW-1 was a long rod (1.5–11 μm long with a diameters of 0.5–1 μm) and had an opaque round colony. Its partial 16S rRNA gene sequences fell within the cluster XI of *Clostridium* genus as described by Collins et al. [44], with *C. bifermentans* as the most closely related species (Fig. 2). HAW-G3 and HAW-G4 were short rods (1.5–3.0 μm long with a diameter of 0.5 μm) and their colonies were transparent and colorless. The 16S rRNA gene sequences of both isolates (HAW-G3 and HAW-G4) fell within the cluster XIVa of *Clostridium* genus [44] and were very closely related to *Clostridium celerecrescens* (ATCC 19403) and *Clostridium sphenoides* (DSM 5628) (Fig. 2). Highest similarity was found between the two isolates (99.8% for HAW-G4; 99.5% for HAW-G3) and *C. celerecrescens*. HAW-E3 (or ES1) was also a short rod (1.5–3.0 μm long with a diameter of 0.5 μm) and its colony was whitish and flat. Its partial 16S rRNA gene sequences fell within the cluster XIVa of *Clostridium* genus [44] with the

Table 2
Growth of bacterial isolates on RDX (0.1 mM) as a nitrogen source and yields of RDX products (measurements were done in triplicates with standard deviations in parentheses)

Isolate	Bacterial growth (OD_{600nm} increase)[a]		Yields of final products (% of total C or N in RDX removed)		
	Without RDX	With RDX	C products		N product
			CH_3OH	HCHO	N_2O
HAW-G4[b]	0.03 (0.02)	0.24 (0.05)	80 (6)	0.47 (0.06)	27 (0.8)
HAW-E3[c]	0.02 (0.01)	0.12 (0.02)	8.7 (0.1)	8.0 (0.4)	33 (2)
HAW-ES2[d]	0.03 (0.01)	0.10 (0.02)	7.5 (0.5)	0.4 (0.1)	35 (2)

[a]Initial biomass (OD_{600nm}): HAW-G4, 0.1; HAW-E3, 0.11; HAW-ES2, 0.07.
[b]Glucose (1 g l^{-1}) was used as a carbon source.
[c]Ethanol (1 g l^{-1}) was used as a carbon source.
[d]Ethanol (1 g l^{-1}) was used as a carbon source and sodium sulfate (3.5 mM) as an electron acceptor.

Clostridium saccharolyticum as the most closely related species (Fig. 2). HAW-HC1 was a long rod (2.5–10 μm long with a diameter of 0.5–1.0 μm) and had an opaque and slightly yellowish colony. Its 16S rRNA gene sequence fell within the cluster I of *Clostridium* genus [44]. Its most closely related species was *Clostridium butyricum* (Fig. 2). The spores in the liquid cultures of the above five clostridial isolates (in yeast extract and peptone medium) could not be killed by heating at 95°C for 10 min. They all fermented glucose and peptone to produce hydrogen gas. The isolate HAW-ES2 was a Gram-negative, non-spore-forming bacterium. Its 16S rRNA gene sequence fell within the cluster of *Desulfovibrio* genus with *Desulfovibrio desulfuricans* as the closest match (Fig. 2). In general, clostridia were found to be the major RDX-removing bacteria.

Beller [12] proposed that homoacetogens are responsible for removal of RDX (at a rate of 0.5 μM day^{-1}) in an aquifer bacterial mixture using hydrogen gas as the electron donor and RDX as a nitrogen source in the presence of carbonate. In the present study, a similar mixed culture removed RDX at a rate of 25 μM day^{-1}, and contained a fast RDX-removing clostridial bacterium HAW-1. A similar clostridial RDX-removing strain identified as *C. bifermentans* was previously isolated by Regan and Crawford [19] from explosive contaminated soil, but the latter appeared to remove RDX at a rate (180 μM day^{-1}, 1.2 OD_{600nm} of biomass) slower than that (330 μM day^{-1}, 0.8 OD_{600nm} of biomass) of isolate HAW-1. To the best of our knowledge, isolate HAW-ES2 is the first RDX-degrading sulfate-reducing bacterium, although a consortium has been reported to remove the energetic chemical under sulfate-reducing condition [13].

Although the presence of methanogens in the ethanol-enriched mixed cultures was demonstrated by the formation of CH_4, we did not obtain any methanogenic isolate from the above ethanol enrichment culture. This could be attributed to the employment of isolation medium that favored the growth of fermentative bacteria rather than methanogens. The present results and the previous reports [10,11,18,19,28,33] showed that most known RDX-removing anaerobic (facultative or obligate) isolates are fermentative bacteria.

3.3. Bacterial diversity on RDX metabolic kinetics

When either glucose (for isolate HAW-G4) or ethanol (for isolate HAW-E3) was used as a carbon source, isolate HAW-G4 and isolate HAW-E3 used RDX as a nitrogen source for growth. Growth of the two isolates on RDX as a nitrogen source was confirmed by an obvious increase in the OD_{600nm} in the presence of RDX and a negligible increase in controls that did not contain RDX or any other nitrogen source (Table 2). The two isolates were also found to grow when RDX in the above media was replaced with the ammonium ion. Addition of yeast extract and peptone improved growth of both isolate HAW-G4 and isolate HAW-E3, and enhanced RDX transformation (data not shown). The specific rate for RDX removal for both isolates (HAW-G4 and E3) was 5.2 and 6.2 μmol h^{-1} g (dry weight) of $cells^{-1}$, respectively. As shown in Table 2, isolate HAW-ES2 also seemed to grow on RDX as a nitrogen source in the basic medium containing ethanol and sulfate (Table 2). The specific RDX-removal rate (5.5 μmol h^{-1} g (dry weight) of $cells^{-1}$) of HAW-ES2 was close to that of isolate HAW-G4 and E3.

Although the enrichment culture was able to grow on RDX and H_2 (Table 1), its isolate HAW-1 did not show any appreciable growth under the same conditions. However, addition of yeast extract greatly improved its growth and the removal rate of the energetic chemical (data not shown). Isolate HAW-1 showed the highest specific RDX-removal rate (24.0 μmol h^{-1} g (dry weight) of $cells^{-1}$) among all isolates, suggesting that this strain might possess a high RDX-transformation activity.

Similarly, the isolate HAW-HC1 from an enrichment culture that was able to grow on RDX as a nitrogen source and formate as a carbon source (Table 1) did not show any obvious growth on RDX under the same conditions. Addition of yeast extract, peptone and glucose moderately improved the growth of strain HAW-HC1 and enhanced RDX removal. Isolate HAW-HC1 exhibited the lowest specific rate (2.5 μmol h^{-1} g (dry weight) of $cells^{-1}$) for RDX removal among all the isolates.

All the above four clostridial isolates are strictly anaerobic fermentative bacteria, exhibiting faster RDX-removal

rates than any previously reported facultative anaerobic bacteria belonging to the fermentative *Enterobacteriaceae* family (10, 11, 18, 28). For example, the RDX-removal rates (2.4–24.0 μmol h^{-1} g (dry weight) of $cells^{-1}$) of the present isolates were approximately 6 to 60 times higher than the rate (0.41 μmol h^{-1} g (dry weight) of $cells^{-1}$) of the previously isolated *K. pneumoniae* strain SCZ-1 [18]. This suggests that obligate fermentative anaerobes are faster in removing RDX than facultative ones.

3.4. RDX metabolic products by bacterial isolates

Like the enrichment cultures, bacterial isolates also transformed RDX to initially produce the nitroso derivatives MNX, DNX and TNX prior to ring cleavage to HCHO and CH_3OH. After 5 days of incubation in the basic salts media containing either glucose or ethanol as a carbon source, isolate E3 and G4 removed 83–99% of 0.1 mM RDX, with production of 8–17% of nitroso derivatives and 3–9.6% of nitrite (relative to the total nitrogen in RDX removed). The yields of TNX of the latter two isolates were less than 1%. During transformation of RDX by isolate ES2, the yields of DNX and TNX were negligible. All the nitroso derivatives were not persistent. Complete removal of RDX and its nitroso derivatives produced the ring cleavage products HCHO and CH_3OH. In one case, isolate HAW-G4 transformed RDX to produce mainly CH_3OH, accounting for 80% of total carbon of RDX removed.

Although we did not detect NO_2^- during RDX incubation with the mixed cultures, we were able to detect it with all isolates with highest yield (9.6% of total N in RDX removed) observed with isolate HAW-E3, indicating that in addition to nitroso routes, RDX degradation also involved denitration. Previously we showed that *Rhodococcus* sp. Strain DN22 aerobically degraded RDX via initial denitration to the dead end product 4-nitro-2,4-diazabutanal [42]. We did not detect the latter in the present study, but we could not exclude its formation probably because of its instability under the anaerobic conditions used.

All isolates produced N_2O as final products, which accounted for approximately one third of the total nitrogen content of RDX removed. The pure cultures fed with NO_2^- in the absence of RDX did not produce N_2O. In contrast to the enriched mixed cultures (Table 1), none of the five isolates removed N_2O, suggesting that other unidentified bacteria in the mixed cultures were responsible for N_2O removal.

The formation of the secondary product N_2O was suggested to be derived from one of the –N–NO_2 groups originally present in RDX [16]. In this paper, all isolates gave N_2O with yields close to 30% of the total nitrogen in RDX (Table 2), suggesting the involvement of only one –N–NO_2 group (representing one third of the total nitrogen content of RDX) in its formation. As reported previously, RDX can be transformed anaerobically via sequential reduction to MNX, DNX and TNX or via initial denitration followed by ring cleavage to produce HCHO, CH_3OH and N_2O [5,18]. In all tested isolates, formation of MNX was found, but TNX and DNX were only detected in low amounts, indicating that denitration of MNX was a dominant route to RDX ring cleavage and secondary decomposition.

All RDX-removing facultative bacteria of the *Enterobacteriaceae* family [10,11,18,28] and strictly anaerobic bacteria [19,33], including the present isolates, are known to contain hydrogenase enzyme [34,35,37–39]. Most of the RDX-removing anaerobic bacterial isolates known thus far are fermentative H_2 producers. Also a rapid H_2-dependent RDX transformation activity was previously described in the crude extract of *Clostridium acetobutylicum* [33]. Finally, *Enterobacteriaceae* only exhibited hydrogenase and RDX-removing activity under anaerobic conditions [10,11,18,28]. Experimental evidence gathered thus far suggests that RDX transformation was initiated by a hydrogenase activity.

Clostridia, which seemingly play a major role in the rapid removal of RDX under strictly anaerobic conditions, are also present in soils contaminated with RDX [19]. Therefore our observations suggest that clostridia in the anoxic environment of soil may also use RDX as a nitrogen source without accumulating nitroso derivatives. On the other hand, aerobic bacteria such as *Stenotrophomonas maltophilia* [40] and species of *Rhodococcus* [41–43] in soil have been reported to degrade RDX. Since both aerobic (surface soil) and anaerobic (subsurface soil) environments are present in soils, both aerobic and anaerobic bacteria will compete for RDX at the oxic/anoxic boundary. Further study on this ecological aspect would be useful to understand how RDX is degraded in a field soil environment.

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